



Lactogenic activity of ethanolic extract of *Pluchea indica* Less leaf in lactating rats



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ABSTRACT

Introduction: The leaves of *Pluchea indica* (L.) Less is empirically used to enhance milk production. This study aimed to evaluate the lactogenic effect of ethanolic extract of *P. indica* leaves (EPI) on milk production, prolactin, cortisol, oxytocin levels, and histological changes of mammary tissue in lactating rats and weight gain of their pups.

Methods: Twenty-five lactating rats with six pups were randomized into five groups. The groups were control (reverse osmosis water), standard (domperidone 2.5 mg/kg BW), and EPI (250, 500, and 750 mg/kg BW). The daily treatments were administered by oral gavage, starting from the second day until day 15 of parturition. Milk production and the weight of the pups were measured daily. Serum prolactin, cortisol, and oxytocin levels in the lactating rats were determined by enzyme immunoassay. Histomorphological alterations of mammary tissues were investigated by hematoxylin and eosin-stained slides. Data were analyzed by one-way ANOVA tests and $P < 0.05$ was considered significant.

Results: The daily milk production in groups treated with domperidone and EPI at doses of 500 and 750 was significantly increased compared to the control group ($P < 0.05$). Weight gain of the pups of dams that received domperidone and EPI at doses 500 and 750 mg/kg BW was significantly higher than controls ($P < 0.05$). EPI 500 insignificantly increased both prolactin and oxytocin, insignificantly decreased cortisol levels, and insignificantly enhanced both numbers of alveoli and branching alveoli with milk secretion ($P > 0.05$).

Conclusion: *Pluchea indica* leaves revealed lactogenic activity in lactating rats.

Implication for health policy/practice/research/medical education:

This research revealed that *P. indica* had a lactogenic effect as evidenced by enhancing of the milk production, weight of the pups, hormones level that affects milk production as well as the numbers of alveoli and branching alveoli with milk secretion. Hence, this plant can be considered to develop as herbal milk booster.

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Introduction

Milk is very important for neonates because it is the only source of food for growth and development until their bodies are able to receive additional food. The production begins between 10-22 gestational weeks (lactogenesis I) and increases immediately between 24-102 hours after birth (lactogenesis II). In the early lactation, breastmilk contains colostrum, a source of passive immune components produced during the first 48 hours after birth, which are not contained in formula milk. Exclusive breastfeeding for 6 months decreased the risk of infants

developing atopic dermatitis, acute otitis media, and hospitalization due to lower respiratory tract infection (1). Accordingly, breastfeeding women who have lactation problems should make efforts to take a synthetic or natural galactagogue to improve milk production.

The antiemetic agents such as domperidone and metoclopramide are used as 'off-label milk boosters' for their galactagogic (lactogenic) effect to stimulate lactation. They are dopamine antagonists, which have the possibility to increase milk production by increasing serum prolactin. Intestinal discomfort and elevation of serum prolactin are

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the most common side effects that occur in infants whose mothers take oral metoclopramide. Depression, vertigo, and headaches were side effects commonly reported by mothers taking metoclopramide, whereas cardiac arrhythmia, cardiac arrest, and death are the reported adverse effects of domperidone. Hence, domperidone has not been approved by the United States Food and Drug Administration (USFDA) as a galactagogue (2,3).

Many communities perceive that medicines from natural resources are safer than synthetic drugs. The leaves of *Pluchea indica* Less also known as *luntas* (Javanese) or *beluntas* (Indonesian, Malaysia) are a natural galactagogue empirically used by breastfeeding women. The leaves are also used as a diaphoretic and febrifuge agent in India and Indo-China (4). The fresh leaves possess an aromatic and astringent taste and are used as a vegetable in South-East Asia (5). The plant is found in India, South-East Asia, and the Philippines (4).

Pharmacological studies have found that *P. indica* inhibits spermatogenesis (6), induces apoptosis of nasopharynx cancer cells (7), has anti-inflammatory (8), antipyretic (9), antibacterial (10), antioxidant (11) and antidiabetic activities (12). *P. indica* produces secondary metabolites such as flavonoids, alkaloids, tannins, and saponins (13). The galactagogic effect of *P. indica* leaves may be related to flavone phytoestrogens (quercetin and kaempferol) found in its leaves (5,14). Phytoestrogenic agents may have effects similar to endogenous estrogen (17 β -estradiol) and have the potential to bind with estrogen receptors that promote proliferation of mammary epithelial cells and thus, secretion of milk (14,15).

Milk production and secretion are influenced by the development of the mammary glands during pregnancy, which are regulated by systemic hormones such as prolactin, estrogen, progesterone, growth hormone, insulin, glucocorticoids, and triiodothyronine (16) and local factors produced by stromal cells, including fibroblast growth factor, insulin-like growth factor-1 (IGF-1), and epidermal growth factor (17). This study aimed to evaluate the effects of *P. indica* on milk production, prolactin, cortisol, oxytocin, histological changes of mammary tissue in lactating rats and weight gain of their pups.

Materials and Methods

The experiments were conducted in the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (UGM) in Yogyakarta, Indonesia. Animals were housed and treated in the Pharmacology and Therapy Laboratory. Hematoxylin and eosin (H&E) staining of mammary tissue was performed in the Department of Anatomical Pathology, FMPHN UGM.

Preparation of plant material

The leaves of *P. indica* were collected from the Yogyakarta Special Region, Indonesia, in March 2020. Identification of this plant was done by a trained botanist from the

Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia and an herbarium specimen was deposited in the Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, UGM (No 12.18.08/UN1/FFA/BF/PT/2020).

Plant extraction

The plant was extracted following a previously used and published method (18). The leaves were dried in the oven at 50°C and then blended to get powder. The powder (200 g) of *P. indica* leaves (EPI) was macerated in 1 L of 70% ethanol for 72 hours. Every 24 hours the extract was filtered using vacuum filtration and the residue was re-macerated twice by renewing the solvent. The filtrates were combined and evaporated to dryness at room temperature.

Animal and experimental design

Mature Wistar albino rats were obtained from the Faculty of Pharmacy UGM. The rats were maintained in an animal room under a controlled environmental condition with temperature 25-26°C, 12/12 of light-dark cycle, and humidity 69-70%. Female rats were housed and mated with male rats in plastic cages and allowed access to food and water *ad libitum*. The food was commercial feed AD-II pellet (from PT Japfa Comfeed Indonesia Tbk.). The day when female rats delivered their pups was designated as day 1 of lactation.

Effect of *Pluchea indica* extract on milk production

A total of 25 lactating Wistar rats weighing 150-250 g (6-8 weeks) were used in this study. Based on Federer's formula to calculate the sample size for several groups, the study population was divided into five experimental groups of five rats each (n=5).

The equation was calculated as follows: $[n-1] (t-1) > 15$, where n=sample size for each group and t=number of interventions. The groups were control (reverse osmosis (RO) water), standard (domperidone 2.5 mg/kg of BW), EPI 250 mg/kg BW, EPI 500 mg/kg BW, and EPI 750 mg/kg BW. Each dam was adjusted to suckle only six pups (18). The animals were treated daily at 18:00 h and treatment was continued from the 2nd to the 15th day of the lactation period. Before giving the treatment, the body weight of the dam and pups were measured. The dams were fed AD-II pellets at 10 g/100 g BW daily, in the morning and evening.

Milk production was measured daily from day 3 to day 15 of lactation by calculating milk yield per pup using the formula from Sampson and Jansen (19). Milk yield per pup = $0.0322 + 0.0667 (\text{weight}) + 0.877 (\text{gain})$. Milk yield is the daily milk yield per pup (g/pup/day), weight is pup weight (g), and gain is pup weight gain per day (g/day). The body weight was measured with an electronic balance (Mettler Toledo) accurate to 0.01 g.

On day 16 of postpartum, blood samples obtained from the retro-orbital sinus were collected for prolactin, cortisol, and oxytocin assays. Then, dams were anaesthetized and euthanized intraperitoneally with rat cocktail solution: 0.125-0.15 mL per 100 g body weight, which contained 25% ketamine 100 mg/mL, 25% xylazine 20 mg/mL, 1% acepromazine 10 mg/mL, and 4% water sterile for injection. The left abdominal mammary glands were dissected and placed in 10% neutral buffered formaldehyde solution for histological analysis.

Histological examination of mammary glands

Mammary gland histology was performed following the standard operating procedures. Mammary glands tissue was dissected, and embedded in formalin-fixed, paraffin-embedded blocks, then sections of 3 μm were cut for each specimen using rotary microtome (20). The sections were deparaffinized with xylene and dehydrated through a graded series of ethanol and stained with H&E. Morphological assessment was performed with Olympus CX23 binocular microscope under 400x magnification. Qualitative and semi-quantitative assessments, including overall morphology, the number and diameter of alveoli, and branching alveoli with milk secretion were counted. Interlobular connective tissue and interlobular excretory ducts in the mammary tissue were also assessed.

Quantitative analysis was determined by means of alveoli number and calculated from a minimum of three regions-of-interest consisted of 5 high-power fields individually. Single-field high resolution digital images were acquired using a single-field high-definition (resolution of 1080p) digital camera (Sigma Optical) mounted to Olympus CX43 binocular microscope. Alveolar diameter measurement was performed to a minimum of 10 alveoli per high-power field images utilizing calibrated line tools in QuPath (open-source software for digital pathology

image analysis) version 0.2.3. The measurement results were converted into microns according to the calibration standard at a resolution of 96 dots per inch (dpi).

Serum prolactin, cortisol and oxytocin levels

The collected blood samples were left at room temperature for 30 minutes and then centrifugated at 2000 g at 4°C for 10 minutes. Serum was separated and stored at -20°C until the prolactin, cortisol, and oxytocin were estimated by enzyme immunoassay. The rat prolactin ELISA kit (Catalog Number RK03905) was obtained from ABclonal. Cortisol EIA kit (Catalog Number CO3685) was purchased from Calbiotech, and General oxytocin ELISA kit (Catalog number RK00696) from ABclonal. The measurements of these serum hormone levels were done in accordance with the manufacturer's instructions.

Statistical analysis

Results were expressed as mean \pm standard error mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc least square difference (LSD), using the statistical package PASW (version 18 for Windows). Results were considered significantly different if $P < 0.05$.

Results

During the experimental period milk production significantly increased over time in all groups ($P < 0.05$). Milk production levels were higher in the groups receiving domperidone or EPI than in the control group. There were significant differences of the milk production from days 7 to 14, in particular for EPI 500 ($P < 0.05$) (Figure 1).

Compared to the control group, the daily milk production per day was significantly increased in groups treated with domperidone and EPI (doses 500 and 750) ($P < 0.05$). The mean daily milk yield was not significantly

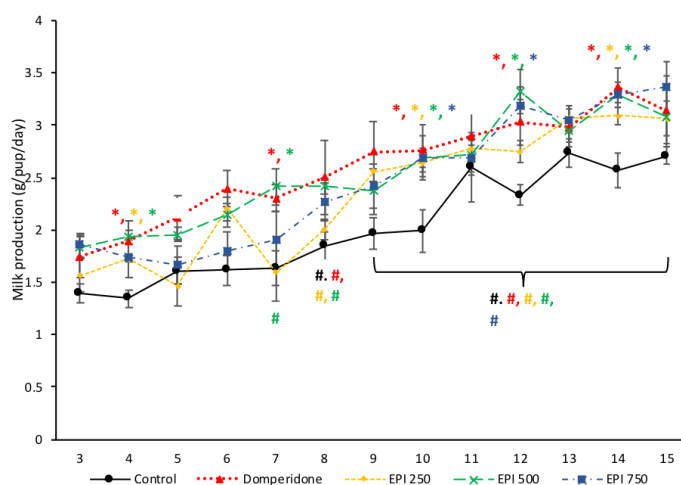


Figure 1. Milk production of Wistar rats receiving the ethanolic extract of *Pluchea indica* during the experimental period. Abbreviations: EPI 250: 250 mg/kg BW of *P. indica* extract, EPI 500: 500 mg/kg BW of *P. indica* extract, EPI 750: 750 mg/kg BW of *P. indica* extract. Values are presented as mean \pm SEM. * and # indicate significantly different ($P < 0.05$) compared to the control group and the third day, respectively (ANOVA followed by LSD).

different between the domperidone- and EPI-treated groups ($P > 0.05$).

Weight gain was significantly higher in the pups from dams that received domperidone and EPI (doses 500 and 750) compared to the control group ($P < 0.05$) (Table 1).

Both the numbers of alveoli and branching alveoli with milk secretion were enhanced in the EPI 500 group compared to the control group ($P < 0.05$). The number of alveoli was significantly higher compared to the domperidone group ($P < 0.05$). There were no significant differences of alveoli diameter among groups although the diameter was increased in both the domperidone and EPI 750 groups compared to the control group. All EPI-treated groups showed a looser interlobular connective tissue compared to the control and domperidone groups. Interlobular excretory ducts were present in the domperidone and all EPI-treated groups but in the control group only one of the five rats presented it (Table 2, Figure 2).

Because milk production, the number of alveoli, and branching alveoli with secretion were the highest in the EPI 500 group and the lowest in the EPI 750 group, then serum prolactin, cortisol, and oxytocin assays were conducted only for the EPI 500 and EPI 750 groups. The results revealed that the serum prolactin levels were insignificantly higher in both the domperidone and EPI 500 groups and lower in the EPI 750 group compared to the control group ($P > 0.05$). The serum cortisol levels were insignificantly lower in both the domperidone and EPI 500 groups compared to the control group ($P > 0.05$) (Figure 3).

The levels of oxytocin in the EPI-treated groups were higher than in both control and domperidone groups

($P < 0.05$), and it was significantly higher in the EPI 750 group compared to the control group ($P < 0.05$) (Figure 4).

Discussion

The present study found that during the experimental period milk production was significantly increased over time in all groups and more in the groups receiving domperidone and ethanolic extract of *P. indica* compared to the control group. In this estimation, the Sampson and Jansen's formula (19) was used, which required the measurement of pup weight and weight gain. The results were in accordance with Liu et al (21) who studied herbal mixture (*Vaccaria hispanica* seed, *Euphorbia heterophylla*, *Astragalus membranaceus*, *Tetrapanax papyriferus*, *Platycodon grandiflorum*, and *Cnicus Benedictus*) and Badgular and Bandivdekar (22) who studied *Cyperus rotundus* Linn. on milk production in lactating rats. There are some factors that are known to affect the level of milk yield over the course of lactation, which are the number of milk-synthesizing epithelial cells, the secretory activity of the epithelial cells, and the supply of nutrients and disposal of waste products through the vascular system (23).

Beside using the Sampson and Jansen's formula, the milk production in lactating rats could be estimated from the difference of pup body weight before and after treatment (24), from mammary gland weight (25) (this method requires excising mammary tissue from the dams), and body water turnover method in rat dams, which requires a dose of labelled water administration to calculate maternal body water (26). This study used the Sampson and Jansen's formula to estimate milk production since it was easier and more practical to apply.

Compared to the control group, the present study

Table 1. Effect of ethanolic extract of *Pluchea indica* on milk production and weight gain of pups

Groups	Milk yield per day (g/pup)	Weight gain of pup per day (g/day)
Control	2.03 ± 0.06	1.21 ± 0.05
Domperidone	2.61 ± 0.17*	1.58 ± 0.14*
EPI 250	2.35 ± 0.07	1.43 ± 0.07
EPI 500	2.55 ± 0.10*	1.47 ± 0.05*
EPI 750	2.46 ± 0.16*	1.47 ± 0.08*

Abbreviations: EPI 250: 250 mg/kg BW of *P. indica* extract, EPI 500: 500 mg/kg BW of *P. indica* extract, EPI 750: 750 mg/kg BW of *P. indica* extract. Values are presented as mean ± SEM. *Significantly different ($P < 0.05$) compared to the control group (ANOVA followed by LSD).

Table 2. Effect of ethanolic extract of *Pluchea indica* on histomorphological alterations of mammary tissue of lactating Wistar rats

Groups	Number of alveoli	Branching alveoli with secretion	Alveoli diameter (µm)	Interlobular connective tissue	Interlobular excretory duct
Control	276.80 ± 3.87	66.20 ± 7.72	274.87 ± 21.13	Present, thin	Only present in one of five rats
Domperidone	210.20 ± 7.74	54.80 ± 3.64	314.95 ± 32.07	Present, thick	Present in all rats
EPI 250	218.20 ± 7.28	64.20 ± 5.05	275.31 ± 22.08	Present, loose	Present in all rats
EPI 500	287.25 ± 47.32*	69.50 ± 4.55	273.24 ± 29.21	Present, loose	Present in all rats
EPI 750	123.75 ± 2.29*	50.00 ± 1.29	292.32 ± 21.17	Present, loose	Present in all rats

Abbreviations: EPI 250: 250 mg/kg BW of *P. indica* extract, EPI 500: 500 mg/kg BW of *P. indica* extract, EPI 750: 750 mg/kg BW of *P. indica* extract. Values are presented as mean ± SEM. *indicates significantly different ($P < 0.05$) compared to domperidone group (ANOVA followed by LSD).

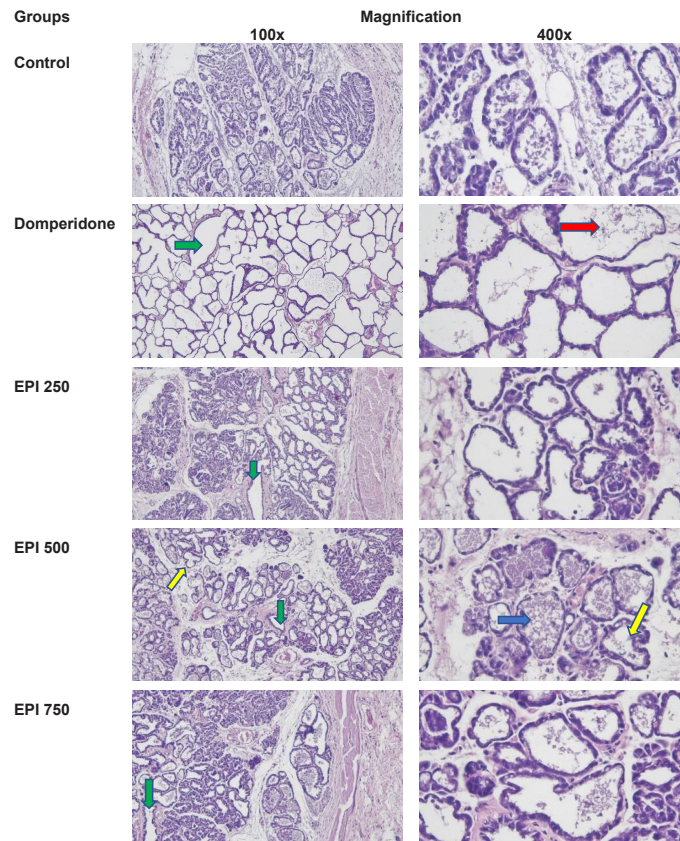


Figure 2. Effect of ethanolic extract of *Pluchea indica* on histological mammary glands of dams stained by hematoxylin and eosin (H&E). Arrows: Alveolus (Red arrow), alveolus with milk secretion (blue arrow), branching alveolus with milk secretion (yellow), interlobular excretory duct (green arrow). Abbreviations: EPI 250: 250 mg/kg BW of *P. indica* extract, EPI 500: 500 mg/kg BW of *P. indica* extract, EPI 750: 750 mg/kg BW of *P. indica* extract.

found that the best lactogenic effect was achieved at the dose of 500 mg/kg BW of the extract (EPI 500). EPI 500 significantly increased both pup weight gain and milk yield. The dose also insignificantly decreased cortisol, increased both prolactin and oxytocin, enhanced both the number of alveoli and branching alveoli with milk secretion ($P > 0.05$). Compared to the domperidone group, the number of alveoli in EPI 500 was significantly higher.

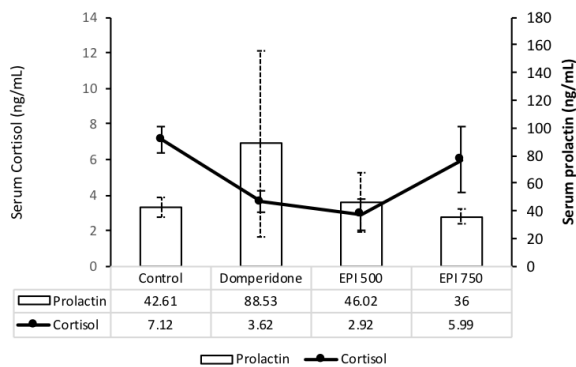


Figure 3. Serum cortisol and prolactin of Wistar rats treated with ethanolic extract of *P. indica*. Abbreviations: EPI 250: 250 mg/kg BW of *P. indica* extract, EPI 500: 500 mg/kg BW of *P. indica* extract, EPI 750: 750 mg/kg BW of *P. indica* extract.

The increasing of pup weight gain may be related to sufficiency of oral intake from milk as proven by increasing milk production from this study. Milk contains carbohydrates as an energy source, proteins, vitamins, minerals, and antibodies which have the main role for the growth and development of offspring. The numbers of alveoli and alveoli with secretion, which represent the number of milk-synthesizing epithelial cells, contribute

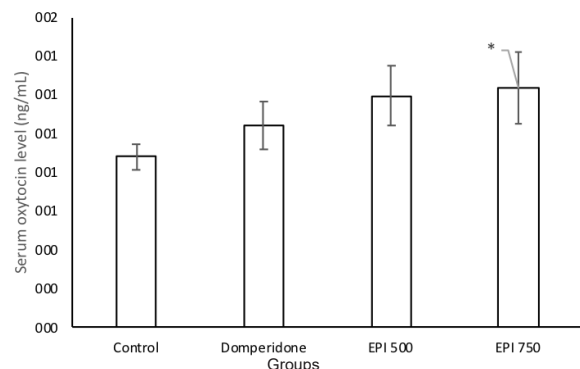


Figure 4. Serum oxytocin level of Wistar rats treated with ethanolic extract of *P. indica*. Abbreviations: EPI 250: 250 mg/kg BW of *P. indica* extract, EPI 500: 500 mg/kg BW of *P. indica* extract, EPI 750: 750 mg/kg BW of *P. indica* extract. Values are presented as mean \pm SEM. * Significantly different ($P < 0.05$) compared to the control group (ANOVA followed by LSD).

to milk yield. Also, EPI 500 insignificantly enhanced the number of alveoli and branching alveoli with milk secretion. Additionally, the number of alveoli in the EPI 500 group was significantly higher compared to the domperidone group.

All EPI-treated groups showed the looser interlobular connective tissue compared to the control and domperidone groups, which may be related to the increased alveoli diameter or size as found in our study. During pregnancy and the lactating period, interlobular tissue in the mammary glands is decreased due to the increased glandular alveoli size (27). Interlobular excretory ducts were present in the domperidone and all EPI-treated groups but in the control group only one of the five rats presented it. This finding suggests that EPI and domperidone may increase the proliferation of luminal epithelial mammary cells.

Milk yield involves the role of the prolactin and oxytocin hormones. Prolactin, a hormone secreted by the anterior pituitary gland, maintains milk synthesis and secretion by acting on the luminal epithelial cells of the mammary gland, and is necessary for survival of alveolar mammary epithelial cells. The secretion is controlled by both stimulatory and inhibitory factors. Oxytocin, and neurotensin, as thyroid releasing hormones act as releasing factors of prolactin. During lactation, suckling and milking stimulate prolactin secretion (28), and dopamine is a dominant inhibitory factor. Stress and fatigue downregulate milk yield because the condition may increase the levels of norepinephrine and dopamine, which then inhibit prolactin synthesis (29). Oxytocin, a hormone secreted by the posterior pituitary gland, is responsible for releasing prolactin and milk ejection. The secretion and release of oxytocin is stimulated by suckling. Suckling stimulates sensory nerve endings in the nipple and areola, and then activates the afferent neural reflexes, which lead to the secretion and release of prolactin and oxytocin (30). Oxytocin contracts the myoepithelial cells of the alveoli and enables milk to be ejected by way of prolactin synthesis (28). Our study found that serum prolactin and oxytocin were insignificantly enhanced in the EPI 500 treated group, and both pup weight gain and milk yield significantly increased. This finding follows the statement by Wan et al (31) that said the increase in serum prolactin levels is not always directly proportional to the amount of milk production. It was proposed that there are mediators and/or other mechanisms that may play a role in milk production in addition to serum prolactin.

The study revealed that the increasing of milk production in the groups receiving the extract of *P. indica* was not different with the domperidone group. It was suggested that they had the same pathway or action mechanism of milk synthesis. Domperidone, a dopamine antagonist, blocks D-2 receptors and induces prolactin synthesis in lactotrophic cells of the anterior pituitary gland. To prove that they have the same pathway, further

studies are needed.

The galactagogue effect of *P. indica* in this study may be related to phytoestrogen ingredients present in this plant. Herbal galactagogues could be mediated by the action of phytoestrogens (32), which may have effect similar to estrogen that stimulates mammary epithelial cells' proliferation (33). Quercetin and kaempferol are phytoestrogens found in *P. indica* leaves (4). Other phytoestrogens contained in herbal galactagogues are diosgenin (*Trigonella foenum-graecum*), shatavarine I (*Asparagus racemosus*), anethole (*Foeniculum vulgare*, *Pimpinella anisum*), silybin A, silybin B, silydianin, and silychristin (*Silybum marianum*). There are many factors that may influence phytoestrogens possessing estrogenic activity. Bioavailability, species of animal, chemical structure of the compound, responsive tissue, and estrogen receptor type are known to affect the activity (15).

The extract of *P. indica* insignificantly decreased cortisol levels. Cortisol has many functions including the control of physiological stress and immunity, and metabolism of carbohydrates, proteins, and fats. Activation of hypothalamic-pituitary-adrenal (HPA)-axis and acute psychological stressor may increase cortisol levels (34). Stress indicated by an increase in cortisol may enhance the incidence of unsuccessful nursing and influence prolactin. Separation from piglets increased cortisol levels in sow (35), and the reduced nursing frequency resulted in lower prolactin levels (36). Stress may suppress lactation by inhibiting prolactin and oxytocin secretion responsible for milk let down (37). The lower cortisol and higher prolactin and oxytocin levels in the EPI groups compared to the control group showed that the rats probably were not in a stressful condition. However, another researcher stated that cortisol was a lactogenic hormone and it should be in sufficient level for milk synthesis (38). So, there is still some controversy concerning the role of cortisol in milk synthesis.

Ethanol extract of *P. indica* enhanced milk yield and the weight gain of pups although without accompanying an increase in prolactin, oxytocin, and lactogenic histological parameters. Probably this happened because the increase in the amount of milk production is not always directly proportional to serum prolactin levels. It was proposed that there are mediators and/or other mechanisms that play a role in milk production in addition to serum prolactin. Somatotropin or growth hormone (GH) is one of those mediators. A previous study investigated that *P. indica* extract increased growth hormone in lactating rats (18). During pregnancy and breastfeeding, GH is required for the growth and development of mammary glands, because it stimulates the proliferation and differentiation of mammary myoepithelial cells, thereby increasing milk production. Other mediators or mechanisms which contribute to increase milk production are daily milking frequency, cell proliferation through IGF-1, cell differentiation through IGF-2, and the activity of

serotonin and parathyroid hormone related protein (39). Thus, many factors have not been studied in the present research, including the quality of the milk, which could influence the growth of the pups. For this reason, hormonal and local factors and also the measurement of the quality of milk can be proposed to be considered in future studies.

Conclusion

Pluchea indica leaves revealed lactogenic activity by increasing the milk production and slightly increasing both prolactin and oxytocin, and the numbers of alveoli and branching alveoli with milk secretion in lactating rats. Therefore, it could be developed as an alternative galactagogue from natural materials. Further researches are needed regarding the safety and appropriate product formulations to ensure the safety.

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Authors' contribution

All authors designed the study. The experiments were conducted by RAS, MMY, and NA. DNMA assessed the H&E-stained slides. NA and RAS analyzed and interpreted the data. RAS and MSHW drafted the manuscript. All authors read and approved the manuscript.

Conflict of interests

No conflict of interest is associated with this work.

Ethical considerations

Animal care and experiments were done in compliance with the guidelines for the care and use of laboratory animals of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. The protocol of the study was approved by the Medical and Health Research Ethics Committee Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada (No KE/FK/0478/EC/2020).

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